Phosphorylation of the hepatic insulin receptor

Stimulating effect of insulin on intact cells and in a cell-free system

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Received 19 April 1982

Hormone receptor *Receptor subunits phosphorylation* *Cell-free system*

1. INTRODUCTION

The current understanding of the molecular basis of insulin action is rather limited despite the impressive effort made by several laboratories to elucidate the mechanisms of action of this hormone [1–3]. However, the first step in insulin action is accepted to be binding of the hormone to cell-surface membrane components termed insulin receptors [4]. Several new techniques have allowed us to define the structural features of the insulin receptor [5–12]. In brief, the insulin receptor is an integral membrane glycoprotein, and has been found, in all tissues studied thus far, to be composed of 2 major subunits (α respectively β) of ~ 135 000 and ~ 95 000 M_r, respectively. All subunits are linked together by disulfide bridges into a large receptor complex of app. M_r 350 000. Both the α-(M_r 135 000) and the β-subunit (M_r 95 000) are glycoproteins. Further, we have shown that both subunits of the insulin receptor are specifically precipitated with autoantibodies against insulin receptor [9–12]. The observation that the entire spectrum of metabolic effects (both acute and late) of insulin can be initiated by the interaction of ligands other than insulin (i.e., autoantibodies to insulin receptor) with the receptor has been taken as evidence that the receptor contains all the necessary information, and/or might be the 'second messenger' for insulin action [13,14]. Further, information has been gathered suggesting that insulin action results in phosphorylation–dephosphorylation reactions in some cellular proteins [3].

Thus an important question arises as to whether the receptor itself possesses an enzymatic activity leading to phosphorylation–dephosphorylation, or is a substrate for such enzymatic activity. In intact rat hepatoma cells and cultured human IM-9 lymphocytes insulin increases the phosphorylation of its own M_r = 95 000 receptor subunit [15].

Here, we have investigated the phosphorylation of the insulin receptor in normal rat hepatocytes, which are major target cells for insulin's actions. We demonstrate in intact hepatocytes that insulin specifically stimulates the phosphorylation of its M_r 95 000 receptor subunit. More importantly, we present the first demonstration of this insulin-induced covalent modification of its receptor subunit in a cell-free system.

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; M_r, relative molecular mass; EGF, epidermal growth factor; TIU, trypsin inhibitor unit

2. MATERIALS AND METHODS

Porcine insulin (monocomponent) was purchased from Novo (Copenhagen); epidermal growth factor was from Collab. Res.; Nonidet P-40, Triton X-100, phenylmethylsulfonyl fluoride, *N*-acetyl-D-glucosamine, wheat germ agglutinin–agarose, collagenase (type I), bovine serum albumin (fraction V), and bacitracin were obtained from Sigma. Adenosine 5′-[γ-^{32}P]triphosphate, tetra (triethylammonium) salt (aqueous solution; 5000 Ci/mmol) and [^{32}P]orthophosphate (carrier-free, 5000 Ci/mmol) were obtained from the Radiochemical Centre Amersham. All reagents for SDS–

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

PAGE were purchased from Biorad Lab. Serum from patient B_2 with autoantibodies to insulin receptor was a kind gift from Dr C.R. Kahn (Joslin Lab., Boston MA) [16–18].

2.1. *Phosphorylation of intact hepatocytes*

Hepatocytes were isolated from male Wistar rats (150–200 g) by collagenase dissociation of the liver according to [19]. After isolation, hepatocytes were washed twice in phosphate-free Krebs Ringer bicarbonate medium enriched with bovine serum albumin (1%, w/v), bacitracin (0.8 mg/ml) and gentamycin (50 μg/ml). Three aliquots of cells (1.5–2 × 10^6 cells/ml) were gassed with 5% CO_2–95% O_2 and incubated in the presence of $^{32}P_i$ (200 μCi/ml) for 30 min at 37°C. Afterwards, to one aliquot of cells, 10^{-7} M insulin and to another aliquot, 5 × 10^{-7} M EGF were added, and the incubation continued for 5 min at 37°C. The reaction was stopped by addition of chilled buffer containing unlabeled phosphate (50 mM), NaF (100 mM), and EDTA (4 mM). The cells were washed twice in this solution, and extracted thereafter with Triton X-100 as in [10]. Insulin receptors were purified 20-fold by chromatography of the cell extract on wheat germ agglutinin–agarose, and then specifically and quantitatively precipitated by autoantibodies to insulin receptor after addition of a second antibody [10]. The immunoprecipitates were collected by centrifugation (10 000 × *g*, 5 min) and washed twice with cold Hepes buffer (50 mM).

2.2. *Phosphorylation procedure for solubilized insulin receptors*

Immediately after isolation, hepatocytes were washed twice with cold buffer containing Tris–HCl (20 mM) (pH 7.4), NaCl (50 mM), bacitracin (1 mM), Aprotinin (1000 TIU/ml) and soybean trypsin inhibitor (10 000 TIU/ml). The cells were collected by centrifugation, resuspended in the wash buffer containing Nonidet P-40 (0.5%) and deoxycholate (0.5%), and homogenized in a Dounce-homogenizer (140 strokes). Thereafter, the broken cells were solubilized for 90 min at 4°C by continuous stirring. This preparation was centrifuged at 100 000 × *g* for 90 min at 4°C, and the insoluble pellet was discarded. The supernatant was applied to a wheat germ agglutinin–agarose column, which was extensively washed at 4°C; desorption of bound glycoproteins was performed using *N*-acetyl-glucosamine (0.3 M). This chromatography allows a 20-fold purification with nearly 100% recovery of the insulin receptor as determined by ^{125}I-insulin binding [10,21]. The two fractions, known to contain insulin receptors [10,21], were pooled and used in the phosphorylation experiments.

For the phosphorylation of solubilized receptors a typical reaction mixture contained the following: solubilized insulin receptors (300 μl); Hepes buffer (20 mM; pH 7.4); $MnCl_2$ (2 mM); 0.125% bovine serum albumin; [γ-^{32}P]ATP (0.1 μM); and, if present, insulin (10^{-7} M), EGF (5 × 10^{-7} M). The final volume was 325 μl. The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP. The reaction tubes were incubated for 5 min at 37°C. The reaction was terminated by addition of 65 μl ice-cold solution containing NaF and EDTA at final concentrations of 100 mM and 10 mM, respectively. The insulin receptors were then quantitatively immunoprecipitated by a serum containing autoantibodies against insulin receptor as in [10]. The immunoprecipitates were analyzed by SDS–PAGE.

2.3. *Gel electrophoresis and autoradiography*

The immunoprecipitates were boiled for 5 min in a solution containing SDS (3%,w/v), glycerol (10%,v/v), sodium phosphate (10 mM), β-mercaptoethanol (2%,v/v) and bromophenol blue (0.01%, w/v). Aliquots of the precipitates were analysed by one-dimensional SDS–PAGE according to [20] using a 5–15% linear gradient of acrylamide as a resolving gel. The gels were stained, dried, and autoradiographed as in [10]. The autoradiograms were scanned in a microdensitometer (Gelman) for quantitative analysis.

3. RESULTS

3.1. *Phosphorylation of insulin receptor in intact hepatocytes*

To investigate whether the insulin receptor can be phosphorylated in hepatocytes, cells were labeled with $^{32}P_i$ in the absence of insulin. When the solubilized labeled glycoproteins obtained from these cells were immunoprecipitated with autoantibodies to receptor, a major labeled band was found with M_r 95 000 (fig.1). This molecular species

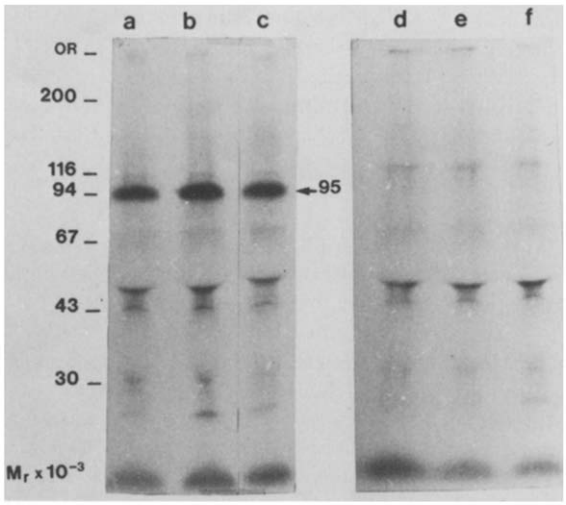

Fig.1. Phosphorylation of M_r 95 000 insulin receptor subunit in intact hepatocytes. Freshly isolated hepatocytes were incubated in $^{32}P_i$ containing medium for 30 min at 37°C. Thereafter, buffer (lane a,d), insulin (10^{-7} M) (lane b,e) and EGF (5×10^{-7} M) (lane c,f) were added for 5 min at 37°C. The reaction was stopped. Insulin receptors were solubilized, purified and subjected to precipitation with either normal serum (lane d–f) or serum containing autoantibodies to insulin-receptor (lane a–c) at a 1:300 dilution as in [10]. The immunoprecipitates were analyzed by SDS–PAGE under reducing conditions. An autoradiogram of a gel is shown.

is recognized as the β-subunit of the insulin receptor because:

(i) Serum from a normal individual does not immunoprecipitate labeled material with the same electrophorectic mobility (fig.1);

(ii) Precipitation of this band by antireceptor serum is decreased when the immunoprecipitation is preceded by exposure to insulin (not shown);

(iii) Using biosynthetic labeling methods in hepatocytes and other cells we have identified a subunit of the insulin receptor with identical electrophoretic mobility [10,21].

Insulin increased significantly the phosphorylation of its receptor subunit (fig.1). Thus, quantitative analysis revealed typically a 30–50% stimulation of the labeling of the M_r 95 000 receptor subunit. Note that insulin had no detectable effect on the $^{32}P_i$ incorporation into total cellular proteins as measured by trichloroacetic acid precipitability.

To test the specificity of insulin's effect, hepatocytes were labeled with $^{32}P_i$ in the presence of EGF, which binds to its own receptor [22] and phosphorylates this receptor in hepatocytes [23]. The stimulating effect of insulin is specific, since EGF was without effect on labeling of the insulin receptor subunit (fig.1).

3.2. *Phosphorylation of purified insulin receptors*

In an attempt to localize the kinase activity responsible for the phosphorylation of the receptor a preparation of soluble insulin receptors was incubated with [γ-^{32}P]ATP in the absence or presence of insulin (10^{-7} M). As observed with intact cells, insulin stimulated the phosphorylation of its own receptor subunit with M_r 95 000 (fig.2). However,

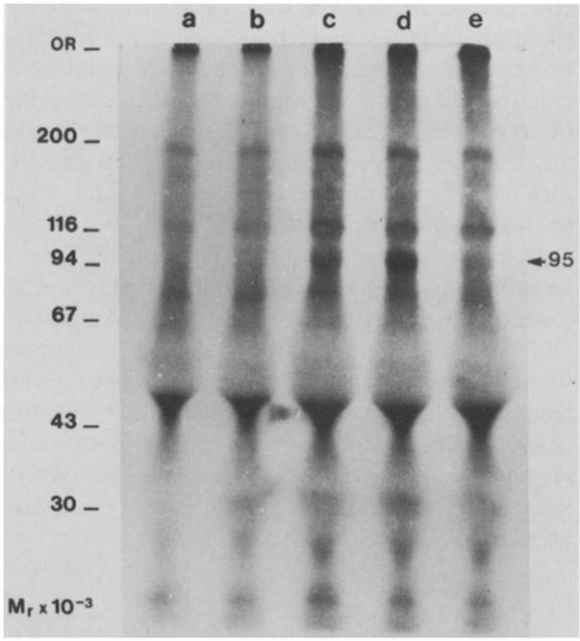

Fig.2. Phosphorylation of M_r 95 000 insulin receptor subunit in a cell-free system. Purified solubilized insulin receptors obtained from normal rat hepatocytes as in section 2, were incubated with [γ-^{32}P]ATP for 5 min at 37°C in the absence (lane a,c) or presence of insulin (10^{-7} M) (lane b,d), EGF (5×10^{-7} M) (lane e). The reaction was stopped. Labeled insulin receptors were immunoprecipitated with serum containing autoantibodies to receptor (lane c–e). In a control experiment a pool of normal sera was used (lane a,b). The precipitates were analyzed by SDS–PAGE under reducing conditions. An autoradiogram of a gel is shown.

insulin's effect was even more pronounced in isolated receptors. Indeed, insulin increased 3–5-fold the incorporation of ^{32}P label in the M_r 95 000 receptor subunit. In contrast, EGF was without effect indicating that insulin's action on its receptor is specific (fig.2).

4. DISCUSSION

We have shown here that in intact normal rat hepatocytes insulin specifically increases the phosphorylation of its receptor subunit with M_r 95 000. This selective stimulating effect of insulin is preserved, and more importantly, is magnified several-fold in a cell-free system using solubilized insulin receptors. The remarkable efficiency of the insulin-stimulated phosphorylation reaction in a cell-free system suggests that the kinase activity is contained within the receptor, or that the receptor and kinase activity are in the same detergent micelle and therefore, are likely to be associated in the intact membrane. At present, the biological role of the insulin-enhanced phosphorylaton reaction of its own receptor is not known. However, it is tempting to speculate on its possible relationship to biologically important processes such as transmembrane hormone signal transfer, hormone-receptor internalization, down-regulation of receptors. We believe that this novel demonstration of insulin receptor phosphorylation in a cell-free system opens a new avenue for investigating the biochemical regulation of the insulin-induced covalent modification of its receptor subunit, and for unraveling the role of receptor phosphorylation in biologically important phenomena.

ACKNOWLEDGEMENTS

We thank A. Le Cam for advice throughout this study and J. Duch for outstanding secretarial assistance.

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